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TITLE: Selection of Aptamers for CED-9/Bcl-2 Family Cell Death Regulations and Their Application in Study of Apoptosis Regulation and Drug Design for Breast Cancer

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14. ABSTRACT Apoptosis has been found to be conserved from <i>C. elegans</i> to humans, suggesting that novel means developed in <i>C. elegans</i> to modulate apoptosis may also be applied to humans for therapeutic treatments of diseases caused by abnormal apoptosis (e.g. cancer, autoimmune diseases, neurodegenerative diseases, etc). In this study, we employed the technique of SELEX to identify small RNA aptamers with high binding specificity and affinity for key cell death regulators, including CED-9 and CED-4 from <i>C.elegans</i> and Bcl-2 and Bcl-xL from humans. So far, we have isolated five RNA aptamers that bind CED-9 with Kds ranging from 5 nM to 10 nM. These aptamers can be categorized into three groups based on their secondary structures and appear to bind to overlapping sites on CED-9. Furthermore, these CED-9 aptamers can form a ternary complex with CED-9 and EGL-1, but not with CED-9 and CED-4, suggesting that these aptamers and EGL-1 bind to different surface regions on CED-9. When over expressed in <i>C. elegans</i> touch receptor neurons, these aptamers can induce ectopic neuronal apoptosis. Importantly, the ectopical cell killing induced by the aptamers is inhibited by a loss-of-function mutation in a key cell-killing gene (<i>ced-3</i>) encoding the caspase, suggesting that these aptamers kill cells through the normal apoptotic pathway. We have conducted several rounds of SELEX experiments on CED-4 and Bcl-xL and obtained candidate molecules with increasing binding affinity <i>in vitro</i> to these two proteins. Our studies suggest that RNA aptamers can be used to modulate apoptosis <i>in vivo</i> and can potentially be used to develop drugs to treat cancers caused by abnormal apoptosis, including breast cancers.					
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Introduction

A central issue in our understanding of cancer biology is how a tissue or organ maintains the appropriate number of cells. As a normal aspect of animal development and homeostasis, programmed cell death (apoptosis) plays an essential role in maintaining the physiological balance of appropriate cell numbers by opposing uncontrolled cell proliferation. Programmed cell death is controlled and executed by a cell death pathway that is highly conserved from the nematode *C. elegans* to humans. At the heart of this pathway is a family of conserved cell death regulators, first defined by the human anti-apoptotic proto-oncogene *bcl-2*. The Bcl-2 family contains both anti-apoptotic and pro-apoptotic members that may regulate the appropriate activation of apoptosis by interacting with and modulating the activities of other cell death regulators or by affecting the membrane permeability of important organelles such as mitochondria. Abnormal inactivation of apoptosis, such as overexpression of *bcl-2*, can lead to uncontrolled cell growth and contribute to the pathogenesis and progression of various human cancers including breast cancer and tumor resistance to chemo- or radio- therapies. Thus, elucidation of the molecular mechanisms by which Bcl-2 family proteins regulate apoptosis is critical for improving our knowledge of cancer biology. Furthermore, identification of small and potent molecular ligands for Bcl-2 family proteins that can be used to activate or inactivate apoptosis at our will can greatly facilitate the development of new therapeutic methods in the treatment and prevention of breast cancer.

Our objective in this study is to carry out *in vitro* selection (SELEX) to identify high affinity and specificity small RNA ligands (aptamers) for the *C. elegans* cell death inhibitor CED-9 (an invertebrate prototype of Bcl-2 proteins) and three important mammalian Bcl-2 family proteins. We will then use *C. elegans* as a key experimental system and isolated aptamers to study how CED-9/Bcl-2 family proteins regulate apoptosis and to screen for potent aptamers that potentially can be applied diagnostically or therapeutically in the detection, prevention, or treatment of human cancer.

Three major goals of this study are: 1) Development of the SELEX method and isolation of aptamers for the *C. elegans* cell death inhibitor CED-9; 2) Characterization of CED-9 aptamers and their effects on *C. elegans* cell death; 3) Isolation and characterization of aptamers for mammalian Bcl-2 family proteins and their effects on mammalian cell death.

We have developed an effective SELEX method to isolate aptamers for CED-9/Bcl-2 proteins. We have used CED-9 aptamers isolated to probe the functional domains of CED-9, the interactions of CED-9 with other cell death regulators, and the mechanistic basis by which CED-9 regulates cell death. We have screened for aptamers that can potentially increase or decrease the activity of CED-9/Bcl-2 proteins *in vivo* and characterized the mechanistic and structural basis of such interference by biochemical and structural biological analyses.

The studies described here will provide novel approaches and generate many new useful reagents for studying the mechanisms of Bcl-2 family proteins in apoptosis, which thus far remain poorly understood. But more importantly, these studies may yield simple and powerful diagnostic reagents for the detection of breast cancer and may generate potent apoptosis-inducing compounds and provide important structural insights for designing new therapeutic drugs in the treatment of breast cancer.

Body:

Task 1. Development of the SELEX method and isolation of aptamers for the *C. elegans* cell death inhibitor CED-9

a. Development of an effective and successful SELEX protocol

This task was completed and described in detail in 2003 annual report.

b. Isolate aptamers for CED-9

Please see the attached manuscript in the Appendix for detailed description

c. Cloning and sequencing of aptamers isolated

Please see the attached manuscript in the Appendix for detailed description

d. Comparison and analysis of the sequences of aptamers and categorization of the aptamers sequenced.

Please see the attached manuscript in the Appendix for detailed description

Task 2. Characterization of CED-9 aptamers and their effects on *C. elegans* cell death.

a. *in vitro* characterization of CED-9 aptamers

Please see the attached manuscript in the Appendix for detailed description

b. The effects of CED-9 aptamers on cell death in *C. elegans*

Please see the attached manuscript in the Appendix for detailed description

Task 3. Isolation and characterization of aptamers for other key cell death regulators including mammalian Bcl-2 family proteins

a. Isolation of aptamers for *C. elegans* pro-apoptotic protein, CED-4

CED-4 is required for the activation of programmed cell death in *C. elegans* (Ellis and Horvitz, 1986). Loss-of-function mutations in *ced-4* result in abolishment of all apoptosis in *C. elegans*. CED-4 is homologous to mammalian pro-apoptotic protein, Apaf-1, which complexes with cytochrome c and dATP to activate procaspase9 (Yuan and Horvitz, 1992; Zou et al., 1997). Genetic studies revealed

that *ced-4* acts upstream of *ced-3* but downstream of *ced-9* during cell death activation (Shaham and Horvitz, 1996). Biochemical studies indicated that CED-4 interacts directly with both CED-3 and CED-9 (Chinnaiyan et al., 1997). However, the molecular mechanisms by which CED-4 activates apoptosis are largely unknown. Therefore, understanding of how CED-4 is regulated by CED-9 and how CED-4 functions to activate CED-3 during apoptosis is critical for understanding of how CED-9 acts to regulate apoptosis and will provide important insights into how Bcl-2

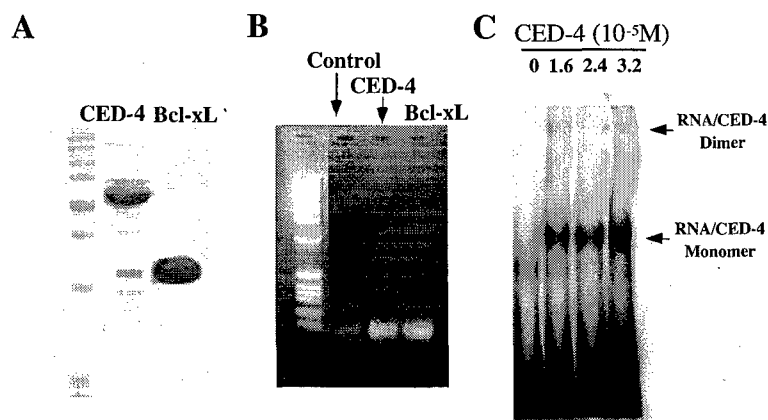


Figure 1. Isolation of aptamers for CED-4 and Bcl-xL. A. Purification of recombinant CED-4 and Bcl-xL. B. Amplification of RNA aptamers for CED-4 and Bcl-xL by RT-PCR (after round 3). C. EMSA of CED4/RNA aptamer complexes.

family proteins function to regulate apoptosis in general. Although isolation of CED-4 aptamers was not suggested in our original proposal, we decided to go ahead to pursue this experiment.

We have expressed and purified the full-length CED-4 protein from bacteria (Fig. 6A). Using this recombinant CED-4 protein, we performed 3 rounds of filter-based SELEX before we turned to EMSA-based selections. As shown in Fig. 6C, we observed 2 shifted bands when increasing concentrations of CED-4 were incubated with RNAs isolated after the 3rd round of filter-based selection. The upper band likely was a complex containing RNA and a CED-4 dimer and the lower band likely was a result of RNA binding to a CED-4 monomer. We have recovered the RNA molecules from these two gel-shift bands and amplified their corresponding cDNAs by RT-PCR. We are in the process of performing 4 additional rounds of EMSA-based SELEX with these RNA molecules. Those RNA aptamers with high affinity for CED-4 will be analyzed further for their abilities to modulate the activity of CED-4 using both *in vivo* and *in vitro* assays.

b. Isolation of aptamers for Bcl-xL, one of the major anti-apoptotic proteins in mammalian Bcl-2 family

Mammalian Bcl-2 family contains both pro- and anti-apoptotic proteins (Vander Heiden and Thompson, 1999). Like Bcl-2, Bcl-xL is one of the major anti-apoptotic proteins that are over-expressed in most cancers, including breast cancer (Thompson, 1995). RNA aptamers with high affinity and specificity for Bcl-xL could be used to inhibit the activity of Bcl-xL and sensitize the cancer cells to chemo- or radio- therapies. Eventually these Bcl-xL aptamers may turn out to be useful for diagnosis or treatment of cancers. We overexpressed and purified recombinant Bcl-xL lacking the C-terminus transmembrane region in bacteria (Fig. 6A). Using this protein, we performed 3 rounds of filter-based SELEX and we were able to detect the enrichment of RNAs binding specifically to Bcl-xL by RT-PCR (Fig. 6B). We are in the process of carrying out several additional rounds of EMSA-based SELEX to isolate RNA aptamers with high binding specificity and affinity to Bcl-xL and will conduct functional analysis of these aptamers using both *in vitro* and *in vivo* assays, as described in the original project proposal.

Key Research Accomplishments

- We have successfully isolated five CED-9 aptamers and obtained sequences of these five CED-9 aptamers
- We found that five CED-9 aptamers can be categorized into three groups and each has a unique secondary structure.
- We demonstrated that two CED-9 aptamers, R9-2 and R9-7, bind to a region in CED-9 that is different from the interacting site between CED-9 and EGL-1 and can form a ternary complex with both CED-9 and EGL-1
- We demonstrated that two CED-9 aptamers, R9-2 and R9-7, and another key pro-apoptotic protein CED-4, bind to overlapping surface regions in CED-9, suggesting that these two aptamers may induce cell death in *C. elegans* by interfering with the binding of CED-9 to CED-4, which inhibits CED-4 pro-apoptotic activity.
- Most importantly, we showed that R9-2 and R9-7 could induce apoptosis in *C. elegans*, suggesting that this SELEX approach can yield molecules that can modulate apoptosis *in vivo* and is likely to have therapeutic potential in treating breast cancer caused by abnormal apoptosis.

- We have conducted four rounds of the SELEX screen for CED-4 aptamers and three rounds of the SELEX screen for Bcl-xL aptamers.

Reportable Outcomes

As discussed above, we not only successfully isolated five aptamers that bind CED-9 with high specificity and affinity *in vitro* but also demonstrated that these aptamers can induce apoptosis in *C. elegans*. Interestingly, these CED-9 aptamers appear to bind to a region in CED-9 that is different from the interacting site between CED-9 and EGL-1 but overlaps with the interacting site between CED-9 and CED-4. These aptamers can form a ternary complex with both CED-9 and EGL-1 but not with CED-9 and CED-4. These findings will provide novel insights regarding how cell death is activated in *C. elegans* and in humans and will provide novel ideas for developing new therapeutic agents to treat breast cancer. We are very excited about these findings and have submitted a manuscript describing our studies of CED-9 aptamers for publication in Proc. Natl. Acad. Sci. USA (please see Appendices). We also presented our studies as meeting abstracts at the 14th International *C. elegans* Meeting at Los Angeles (2003) and the 4th Era of Hope Meeting at Philadelphia (2005) (please see Appendices). Dr. Chonglin Yang, a postdoctoral trainee supported by this grant, obtained a faculty position in the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Dr. Jay Parrish, a graduate student who initiated the work supported by this grant, is currently a postdoctoral fellow at the University of California at San Francisco. Mr. Nathan Camp, a Professional Research Assistant supported by this grant, was accepted into the graduate program in the University of Washington at Seattle.

Conclusions

We have developed an effective SELEX method to isolate high affinity and specificity small RNA ligands (aptamers) for a target protein. Using this method, we have successfully isolated five aptamers for the *C. elegans* cell death inhibitor CED-9. Based on the sequences of these aptamers and their predicted secondary structures, these aptamers can be categorized into three groups. Our *in vitro* biochemical analyses indicate that these CED-9 aptamers bind to a region in CED-9 that is different from the interacting site between CED-9 and EGL-1 but overlaps with the interacting site between CED-9 and CED-4. These aptamers can form a ternary complex with both CED-9 and EGL-1 but not with CED-9 and CED-4. Most importantly, we showed that two of the CED-9 aptamers, R9-2 and R9-7, could strongly induce apoptosis in *C. elegans*, suggesting that this SELEX approach can yield molecules that can modulate apoptosis *in vivo*. We have also made significant progresses and obtained promising results on our efforts to isolate aptamers for a key *C. elegans* cell death activator CED-4 and aptamers for Bcl-xL, a mammalian homologue of CED-9 and a key mammalian anti-apoptotic protein. Once the aptamers for CED-4 and Bcl-xL are isolated, we will use these aptamers to probe the interactions of CED-4/Bcl-xL with other apoptotic regulators and the functioning mechanisms of CED-9/Bcl-2 family proteins in regulating apoptosis. We will further explore the possibilities that some of these aptamers can be used to perturb (induce or block) apoptosis in human cells and thus can be used or modified to become potential diagnostic or even therapeutic agents for the detection or treatment of cancers, which most often are caused by inappropriate apoptosis (Thompson, 1995). A research grant support from the U.S. Army Medical Research and Materiel Command is critical in helping us complete these research achievements.

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Appendices

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2. Chonglin Yang, Nieng Yang, Xiaochen Wang, Jay Parrish, Yigong Shi, And Ding Xue (2005). Selection of aptamers for CED-9/Bcl-2 family cell death regulators and their application in study of apoptosis regulation and drug design for breast cancer. The 4th Era of Hope Meeting Abstracts.
3. Yang, C.L., Yan, N., Parish, J.Z., Wang, X.C., Shi, Y.G., and Xue, D. (2005). RNA aptamers targeting the cell death inhibitor CED-9 induce cell killing in *C. elegans*. Manuscript submitted to Proc. Natl. Acad. Sci. USA.
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Abstract presented at the 14th International *C. elegans* meeting at Los Angeles in 2003.

Modulation of Programmed Cell Death with RNA Aptamers

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Programmed cell death (apoptosis) plays an essential role in maintaining the physiological balance of appropriate cell numbers by opposing uncontrolled cell proliferation. The pathway of programmed cell death appears to be highly conserved from *C. elegans* to mammals, suggesting that the studies of programmed cell death in *C. elegans* can provide important information for understanding how cell death is regulated and executed in mammals. More ever, novel ways developed in *C. elegans* to modulate programmed cell death may also be applied to mammals for better detection, prevention as well as treatment of human diseases such as cancer caused by abnormal apoptosis. In this study, we are employing the technique of SELEX (Systematic evolution of ligands by exponential amplification) to identify small RNA aptamers with high binding specificity and affinity to key cell death regulators including CED-9 and CED-4 from *C.elegans* as well as Bcl-2 and Bcl-xL from humans. We hope to use these RNA aptamers to probe how Bcl-2 family proteins regulate programmed cell death in both *C. elegans* and mammalian cells. More importantly, modulating the process of programmed cell death in *C. elegans* using these specific RNA aptamers may provide important insights into devising new diagnostic and therapeutic drugs for various cancer treatments. So far 6 rounds of SELEX have been performed on CED-9, CED-4 and Bcl-xL and we have obtained candidate molecules with increasing RNA binding affinity *in vitro* to these proteins. The potential effects of individual RNA molecules on apoptosis will be investigated in detail both *in vitro* and *in vivo*, after more rounds of selection are performed.

Abstract presented at the 4th Era of Hope meeting at Philadelphia organized by the Department of Defense (DOD) Breast Cancer Research Program (BCRP).

Selection of aptamers for CED-9/Bcl-2 family cell death regulators and their application in study of apoptosis regulation and drug design for breast cancer

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Small chemical compounds capable of binding to important biomolecules with high specificity and affinity can be used to modulate the activities of these biomolecules and the underlying biological processes. In this study, we have developed an effective SELEX method (systematic evolution of ligands by exponential amplification) to isolate small RNA ligands (aptamers) that bind crucial cell death regulators with high affinity and specificity. Using this method, we have successfully isolated five aptamers for the *C. elegans* cell death inhibitor CED-9. These five aptamers can be categorized into three groups based on their RNA sequences and predicted secondary structures. In vitro biochemical analyses indicate that these aptamers have high CED-9 binding affinities (Kds ranging from 5 nM to 10 nM) and recognize a region in CED-9 that is different from the interacting site between CED-9 and EGL-1 (a *C. elegans* cell death initiator). Consistent with these observations, these CED-9 aptamers can form a ternary complex with CED-9 and EGL-1. In contrast, these aptamers appear to recognize a part of the binding surface between CED-9 and CED-4 (another *C. elegans* pro-apoptotic protein and a CED-3 cell death protease activator) and can interfere with CED-4/CED-9 binding. Indeed, two of the aptamers tested can mimic EGL-1 in releasing CED-4 from the CED-9/CED-4 complex, a crucial step in the cell death activation in *C. elegans*. Most importantly, these two CED-9 aptamers can potently induce apoptosis in *C. elegans*, suggesting that the SELEX method can yield molecules that can modulate apoptosis in vivo. We have made significant progresses in isolating aptamers for CED-4 and Bcl-xL, a mammalian homologue of CED-9 and a key mammalian anti-apoptotic protein. We will use these aptamers to probe the interactions of CED-4 or Bcl-xL with other apoptotic regulators and the functioning mechanisms of the CED-9/Bcl-2 family proteins in regulating apoptosis. We will further explore the possibilities of applying these aptamers to induce or inhibit apoptosis in human cells. Ultimately we hope that these aptamers will be basis for generating diagnostic or therapeutic agents for the detection or treatment of cancers or other human diseases caused by inappropriate apoptosis.

Biological Sciences/Biochemistry

RNA aptamers targeting the cell death inhibitor CED-9 induce cell killing in *C.*

elegans

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Twenty-five text pages, four figures and two tables

Abstract: 185 words

Paper: 37569 characters

Bcl-2 family proteins include anti- and pro- apoptotic factors that play important roles in regulating apoptosis in diverse species. Identification of compounds that can modulate the activities of Bcl-2 family proteins will facilitate development of drugs for treatment of apoptosis-related human diseases. We have used SELEX to isolate RNA aptamers that bind the *C. elegans* Bcl-2 homolog CED-9 with high affinity and specificity and have used these aptamers to modulate programmed cell death in *C. elegans*. Five CED-9 aptamers were isolated and classified into three groups based on their predicted secondary structures. Biochemical analyses indicate that two of these aptamers, R9-2 and R9-7, and EGL-1, an endogenous CED-9-binding pro-apoptotic protein, bind to distinct regions of CED-9. However, these two aptamers share overlapping CED-9 binding sites with CED-4, another CED-9-binding pro-apoptotic protein. Importantly, ectopic expression of these two aptamers in touch receptor neurons induces efficient killing of these neurons in a CED-3 caspase-dependent manner. These findings suggest that RNA aptamers can be used to modulate programmed cell death *in vivo* and can potentially be used to develop drugs to treat human diseases caused by abnormal apoptosis.

Apoptosis is an essential cellular process that is critical for tissue homeostasis and animal development in metazoans. Abnormal inactivation of apoptosis can result in uncontrolled cell growth, leading to development of cancer and autoimmune disorders. By contrast, inappropriate activation of apoptosis can cause too much cell death, leading to neurodegenerative diseases and immunodeficiency (1, 2). Development of effective

therapeutic methods that can correct or reverse inappropriate apoptosis is thus a critical issue in clinical medicine.

Apoptosis is controlled and executed by an evolutionarily conserved cell death pathway (3, 4). At the center of this pathway is a family of conserved cell death regulators, first defined by the human proto-oncogene *bcl-2*, which promotes cell survival and was identified by virtue of its overexpression in a number of B-cell lymphomas (5-7). Subsequently, a family of Bcl-2 related proteins, characterized by the presence of at least one of four conserved Bcl-2 homology (BH) domains, has been discovered and found in organisms as distantly related as *C. elegans* and humans (8). Members of this family can be either anti-apoptotic or pro-apoptotic and can form heterodimers with selected family members to affect apoptosis. The mechanisms by which Bcl-2 family proteins regulate cell death appear to be quite complicated but likely involve modulation of the mitochondrial permeability and the release of crucial apoptogenic factors such as cytochrome c, AIF, and endonuclease G, which promote activation of caspases, the cell death executors, and other cell death events such as chromosome fragmentation (8, 9). In addition to B-cell lymphomas, Bcl-2 family members are overexpressed in a wide variety of cancers, which contributes to malignant growth of tumors as well as tumor resistance to chemotherapies (10). Thus Bcl-2 family proteins are ideal targets for pharmaceutical intervention in the treatment of cancer and other human diseases.

Genetic studies in *C. elegans* have identified a central cell killing pathway involving four genes (*egl-1*, *ced-9*, *ced-4*, and *ced-3*) that act in a negative regulatory cascade to control activation of programmed cell death (4). Biochemical studies indicate that EGL-1, a BH3-only proapoptotic protein, induces cell death by binding to and

inhibiting the activity of CED-9, a cell death inhibitor and a homologue of human Bcl-2, leading to the disassociation of CED-4 from the CED-4/CED-9 complex tethered on the surface of mitochondria (4). CED-4, a homologue of the human Apaf-1 protein (apoptotic protease activating factor), then activates the CED-3 caspase and apoptosis through an unknown mechanism (4). As in humans, misregulation of apoptosis in *C. elegans* can have detrimental outcomes. For example, loss-of-function mutations in the *ced-9* gene cause embryonic lethality as a result of too much cell death (11, 12). Importantly, key components of this cell death pathway are highly conserved from nematodes to humans (4), indicating that studies of apoptosis in *C. elegans* will be highly relevant to studies of apoptosis in humans.

Small RNA molecules play critical roles in regulating many important cellular events such as ribosome biogenesis, RNA splicing, gene silencing (RNA interference), and protein translation (microRNA) (13) and can potentially be used as therapeutic agents. During past decade, an *in vitro* selection method named SELEX (systematic evolution of ligands by exponential enrichment) has been developed to isolate small RNA molecules (aptamers) that have high binding affinity and specificity to important biomolecules such as proteins and RNAs (14, 15). Some of the aptamers have been used to treat human diseases caused by misexpression or altered activity or function of their target proteins. For example, aptamers with high affinity for vascular endothelial growth factor (VEGF) are being clinically used to treat blindness caused by macular dysfunction (16, 17). Aptamers targeting blood coagulation factors VIIa and IXa are promising anticoagulants (18, 19). In addition, aptamers have also been developed to modulate the activities of other proteins critical for various biological processes, including

transcriptional factor NF-Kappa B (20-22) and E2F (23), epidermal growth factor (24), *Drosophila* nuclear splicing factor B52 (25, 26), and others (27-30). Thus SELEX could be an effective method to identify new small molecule regulators for Bcl-2 family proteins.

In this study, we have used SELEX to identify RNA aptamers for the *C. elegans* cell death inhibitor CED-9 and have isolated five different CED-9 aptamers, which can be categorized into three different groups based on their predicted secondary structures. We found that two of these aptamers, R9-2 and R9-7, and EGL-1 bind to different regions of CED-9 but seem to have overlapping CED-9 binding sites with CED-4. Importantly, overexpression of these two CED-9 aptamers induces ectopic cell killing in *C. elegans* touch receptor neurons, and this ectopic killing is suppressed by a strong loss-of-function mutation in the *ced-3* gene. Therefore, these aptamers may antagonize the pro-survival activity of CED-9 and kill cells through the normal programmed cell death pathway. Our results suggest that RNA aptamers for key cell death regulators can yield, or serve as leads to generate, potent compounds to modulate apoptosis *in vivo*.

Material and Methods

Recombinant proteins

CED-9(1-251)-His₆ was expressed in bacterial BL21(DE3) cells and purified with Ni-NTA beads according to the instruction of the supplier (Qiagen). After purification, the recombinant proteins were dialyzed in a buffer containing 25 mM Tris-HCl (pH7.5), 50 mM NaCl, and 10% glycerol. Recombinant GST-EGL-1, GST-CED-9 (wild type and mutants), and CED-4 were purified as previously reported (31).

Systematic evolution of ligands by exponential enrichment (SELEX)

The RNA library for the first round of SELEX was generated from a DNA template containing 49 randomized nucleotides flanked by fixed linker regions and a T7 promoter for *in vitro* transcription (Table 1). RNA was transcribed overnight and purified from 10% denature acrylamide gels. For the first 6 rounds of selection, a filter-binding assay was used. Briefly, RNA was pre-incubated with a nitrocellulose filter (HAWP 0.45um, Millipore) for one hour at room temperature to reduce non-specific binding of RNA molecules. Then the counter-selected RNA was incubated with CED-9(1-251)-His₆ at 30°C for 30 min in a buffer containing 10 mM Tris-HCl (pH7.5), 1 mM DTT, 50 mM NaCl, and 10% glycerol. The reaction mix was subsequently filtered gently through the nitrocellulose membrane with a filtration apparatus and the membrane was washed 4 times with 1 ml washing buffer (20 mM HEPES-KOH pH8.0; 0.2 mM EDTA, 100 mM KCl, and 20% glycerol). The membrane containing the protein-RNA complex was incubated with proteinase K at 55°C for 20 min in a buffer containing 100 mM Tris-HCl (pH8.0), 12.5 mM EDTA, 150 mM NaCl and 1% SDS, followed by phenol:chloroform extraction, and precipitation with cold ethanol. The amounts of protein and RNA used for binding were gradually decreased with the increasing rounds of selection. An electrophoretic mobility shift assay was used for the last three rounds of SELEX. About 300 cpm ³²P labeled RNA was incubated with different amount of CED-9(1-251)-His₆ in a reaction mix of 20 µl for 30min at 30°C in the same protein-RNA binding buffer as above and separated with 7.5% native polyacrylamide gels. The shifted protein-RNA complex was cut off the gel and the RNA was recovered as described above. For both selection assays, the selected RNA was reverse-transcribed into single-stranded cDNA

with AMV reverse transcriptase at 42°C for 1 hour according to the instructions from the provider (Fisher BioReagents). The cDNA was further amplified by PCR using the primers corresponding to the fixed regions at both ends; and the amplified DNA was subjected to next round of *in vitro* transcription and selection. The RNA selected after round 8 and round 9 was amplified by RT-PCR and ligated into the *C. elegans* expression vector pPD52.102 via its Nhe I and EcoR V sites. After transformation of the ligation products into DH5 α cells, PCR was performed on the transformant colonies to amplify the inserts, which were digested with the 4 base-cutter enzyme Aci I for fingerprinting on 4% agarose gel. The binding of individual aptamers to CED-9 was further confirmed by gel-shift assays and aptamers with high binding affinity to CED-9 were sequenced. The secondary structures of these aptamers were predicted with the program MFOLD (32).

Electrophoretic mobility shift assay (EMSA)

CED-9(1-251)-His₆ or GST-CED-9 protein was incubated with ³²P-labeled aptamers (about 150 cpm) in a 20 μ l reaction mix in binding buffer (as described above) supplemented with 2 μ g of yeast tRNA as a non-specific binding competitor. After incubation at 30°C for 30 min, the reaction mix was separated on 7.5% native polyacrylamide gel at 4°C. For the competition binding assay, indicated amounts of unlabeled aptamer were added to a reaction mix with a fixed concentration (0.5 μ M) of CED-9(1-251)-His₆. Both ³²P labeled and cold aptamers were denatured at 85°C for 5 min and cooled on ice for 5 min before being added to the reactions.

Overexpression of aptamers in *C. elegans*

The cDNAs encoding the aptamers with high binding affinity to CED-9 were inserted into the *C. elegans* expression vector pPD52.102 via its Nhe I and EcoR V sites, which directs the expression of the aptamer in six touch receptor neurons under the control of the promoter of the *mec-7* gene. The aptamer expression vectors were linearized with EcoRV and injected into wild-type animals with the co-injection marker pRF4, which causes the Roller phenotype. Two to four independent transgenic lines were scored for the phenotypes caused by the overexpression of each aptamer.

Results

Isolation and characterization of CED-9 aptamers

In order to identify RNA aptamers for CED-9, SELEX was carried out using a protocol described by Chen et al. (24). Briefly, an RNA library was generated *in vitro* using an oligonucleotide library that contains a central region of 49 randomized nucleotides flanked at both ends by constant sequences and a bacterial T7 promoter for *in vitro* transcription (Table 1). For the first round of SELEX approximately 10^{15} unique sequences were represented. Each round of SELEX consisted of the following steps: radioactive-labeled RNAs were incubated with purified recombinant CED-9 before the reaction mixes were applied to a protein binding assay such as the electrophoretic mobility shift assay (EMSA) or the filter binding assay. CED-9/RNA complexes that were isolated from the EMSA or retained by the filter (Materials and Methods) were recovered and reverse transcribed to cDNAs, which were then PCR-amplified to generate a new oligonucleotide library enriched in DNAs encoding RNAs with higher binding

affinity for CED-9. After nine rounds of SELEX, we obtained a pool of RNA molecules that bound CED-9 with high affinity (see below).

To determine the sequences of isolated CED-9 aptamers, we cloned the corresponding cDNA molecules of aptamers obtained from the last two rounds of SELEX. Initially, we checked these cloned cDNA molecules with restriction enzyme finger printing to determine the relative abundance of closely related aptamers in these pools (Materials and Methods). To do this, the RNA coding regions of 30 cDNA clones from each round of SELEX were PCR amplified and digested with a frequent four cutter, Aci I. Clones with the same restriction digestion pattern were assumed to have identical or very close DNA sequences. From this analysis, we were able to identify several different aptamers. We then tested their binding affinities for CED-9 using EMSAs and chose for further analysis the aptamers that bound CED-9 with the highest affinity. Five aptamers obtained from these secondary selections are shown in Fig. 1A and Table 1.

Among the five aptamers, R8-20 was isolated from the round 8 SELEX and the other four were isolated from round 9. Based on the restriction enzyme finger printing analysis, these aptamers are likely the most abundant RNAs in the last two rounds of SELEX. Therefore further rounds of selection would not likely yield significantly different pools of aptamers. Two of the five aptamers, R8-20 and R9-7, differ by only 3 nucleotides (Table 1). Since these two aptamers were isolated from consecutive rounds of selection, our SELEX method appears to be able to enrich specific binders of CED-9, as shown by increased frequency of their isolations (3/30 for R8-20 and 9/30 for R9-7, respectively, Table 1). EMSAs indicate that all these aptamers bound CED-9 very well (Fig. 1A). Interestingly, R9-2 has slightly higher CED-9 binding affinity than the other

aptamers, although the frequency of R9-2 isolation is not as high as those of the other aptamers (Fig. 1A and Table 1). It is likely that R9-2 will be enriched with more rounds of selection.

To further characterize these CED-9 aptamers, we used the Mfold program to predict secondary structures of these aptamers. Based on the predictions, these five aptamers can be categorized into 3 groups. Group A includes R8-20, R9-7 and R9-4, which have very similar trifoliate stem-loop structures. Group B has R9-2 and Group C includes R9-8, both of which contain rod-like stem-loop structures (Fig. 1B). The different predicted secondary structures of these aptamers suggest that they may have different CED-9-binding properties. Again, the similarity of secondary structures within each aptamer group indicates that our SELEX enriched specific aptamers for CED-9. In the studies described below, we chose R9-7 from group A, which is relatively more abundant, and R9-2 from group B, which has very good binding affinity to CED-9, for more detailed analyses.

R9-2 and R9-7 aptamers bind to overlapping sites on CED-9

We first determined the binding affinity of R9-2 or R9-7 to CED-9 using competition-based EMSA. In these assays, ³²P-labeled aptamer/CED-9 complexes were incubated with increasing amounts of unlabeled aptamer and the amounts of labeled aptamer that remained in aptamer/CED-9 complexes were monitored. The disassociation constant (*K_d*) of CED-9/R9-2 complexes measured using this assay is approximately 4 nM, while the *K_d* for R9-7/CED-9 complexes is approximately 16 nM (data not shown). Based on the Mfold predictions, R9-2 and R9-7 have different secondary structures and

may bind to different surface regions of CED-9. We therefore tested whether these two aptamers bind to the same surface regions of CED-9 by performing a modified competition EMSA, in which unlabeled R9-7 was incubated with ^{32}P -labeled R9-2/CED-9 complexes or vice versa. As shown in Fig. 2A, R9-7 can compete with R9-2 for binding to CED-9, although at least 4 fold excess of cold R9-7 was required for this competition. Similarly, R9-2 could efficiently compete away the binding between R9-7 and CED-9 but much less cold R9-2 was needed for the competition (Fig. 2B). These results suggest that R9-2 and R9-7 may contact CED-9 at overlapping sites. However, it is also possible that the binding of one aptamer to another CED-9/aptamer complex could induce a conformational change in CED-9 that causes dissociation of the other aptamer. In such a scenario, the two aptamers could bind to different regions of CED-9 but still effectively compete with each other for CED-9 binding. To test this possibility, we performed limited proteolytic digestion of CED-9 or CED-9/aptamer complex with trypsin and monitored the digestion patterns using gel electrophoresis. The CED-9 trypsin digestion patterns were essentially the same with or without aptamers (data not shown), suggesting that no obvious conformational change of CED-9 was induced by the binding of the aptamers. In contrast, binding of EGL-1 to CED-9 induces conformational changes of CED-9 (31) and results in a different trypsin proteolysis pattern of CED-9 (data not shown). Taken together, these observations suggest that these two aptamers bind to the same or overlapping regions on CED-9, despite having different predicted secondary structures.

The effects of aptamers on CED-9/EGL-1 and CED-9/CED-4 complexes

In *C. elegans*, CED-9 functions as an anti-apoptotic factor. It has been suggested that CED-9 inhibits cell death by binding to and tethering the pro-apoptotic protein CED-4 onto the surface of mitochondria (33-35). In cells that are doomed to die, the cell death initiator EGL-1 is transcriptionally up-regulated and then binds to CED-9, resulting in the release of CED-4 from the CED-9/CED-4 complex and translocation of CED-4 from mitochondria to the perinuclear region, where it promotes the activation of the CED-3 caspase via an unknown mechanism (35). To evaluate the potential effects of CED-9 aptamers on cell death, we tested if CED-9 aptamers interfere with the interactions of CED-9 with EGL-1 and CED-4. Using EMSA, we found that EGL-1 could form a ternary complex with CED-9/R9-2 or CED-9/R9-7 as indicated by the supershifted CED-9/aptamer complexes following addition of EGL-1 (Fig. 3A and B). Since EGL-1 alone does not interact with either of these aptamers, these results suggest that EGL-1 and R9-2 (or R9-7) likely bind to different regions of CED-9. In contrast, recombinant CED-4 failed to super-shift the CED-9/aptamer complexes (Fig. 3C), suggesting that CED-4 probably could not form higher order complexes with CED-9 and its aptamers.

Some CED-4 binding surfaces on CED-9 are important for aptamer binding

Our results indicate that R9-2 and R9-7 can form ternary complexes with CED-9/EGL-1 but not with CED-9/CED-4. To identify the CED-9 binding areas of these two aptamers, we tested the binding of these two aptamers to several CED-9 proteins that contained amino acid substitutions in surface exposed residues (31). As shown in Fig. 4A, the majority of CED-9 mutations that affect the binding between CED-9 and R9-2 also affect the binding between CED-9 and R9-7, confirming that these two aptamers share

overlapping CED-9 binding sites. Since R9-2 and R9-7 have different secondary structures, they likely contacted some distinct surface areas of CED-9. Consistent with this hypothesis, CED-9(P103G, G104E), CED-9(E136K, Q137A), CED-9(KKH125-128EEA), and CED-9(Y201D) showed different binding affinities to these two aptamers (Fig. 4A). Importantly, three sets of CED-9 mutations (NAQ158-160AGA, Y201D, and RN211-212EG) that disrupted binding of CED-9 to both aptamers are also critical for CED-4/CED-9 interaction (31)(Yan and Shi, unpublished data). Thus, these three CED-9 surface areas are important for both CED-4 and aptamer binding. Together with the EMSA results shown in Fig. 3C, these findings suggest that R9-2, R9-7, and CED-4 share overlapping binding sites on CED-9.

We further tested if those CED-9 mutations that affected CED-9 binding to aptamers also affected CED-9/EGL-1 interaction. Using the glutathione-S transferase (GST) fusion protein pull-down assay, we found that wild type and the mutant GST-CED-9 proteins bound equally well to ³⁵S-Methionine-labeled EGL-1 proteins, indicating that none of the CED-9 mutations that reduced or abolished the aptamer binding to CED-9 significantly affected the interaction between CED-9 and EGL-1 (Fig. 4B). These results are consistent with the observations that EGL-1 and aptamers bind to different surface areas of CED-9 and provide further confirmation to the finding derived from the structural analysis that EGL-1 and CED-4 bind to distinct surface areas of CED-9 (31).

CED-9 aptamers induce ectopic cell killing in *C. elegans*

Our *in vitro* studies indicate that two of the CED-9 aptamers, R9-2 and R9-7, bind to overlapping regions on CED-9, which appear to be different from the EGL-1 binding

sites on CED-9. Furthermore, our data indicate that these aptamers and CED-4 likely share some binding surfaces on CED-9 and the binding of aptamers to CED-9 may interfere with CED-4/CED-9 interaction. We thus tested if these aptamers could promote cell killing *in vivo* by ectopically expressing CED-9 aptamers in touch receptor neurons under the control of the promoter of the *mec-7* gene (36, 37).

We first generated transgenic lines with low copy extrachromosomal arrays expressing aptamer R9-2 or R9-7. In these transgenic animals, R9-2 caused approximately 30% killing of the PLM touch receptor neurons (68 and 74% PLM survival in two different transgenic lines) (Table 2). Similarly, R9-7 caused approximately 15% of PLM killing (83 and 85% survival). In contrast, a control aptamer that did not bind CED-9 had very low PLM killing activity (93 and 98% PLM survival) (Table 2 and data not shown). The difference between R9-2 and R9-7 in cell killing is consistent with the finding that R9-2 has a higher binding affinity to CED-9 than R9-7 (Fig. 2).

In *C. elegans*, the expression levels of a protein from transgenes normally correlate with the numbers of copy of a gene present in transgenes (38). To test if these two aptamers can kill cells in a concentration-dependent manner, we generated high-copy number transgenes expressing these two aptamers and found that they killed the touch receptor neurons equally well, resulting in approximately 80% killing of the PLM neurons (Table 2). The PLM killing activities of these two aptamers were comparable to that of EGL-1, which is a potent cell death inducer (Table 2). In contrast, the same control aptamer expressed in high copy transgene arrays had a marginal cell killing activity (Table 2). The low percentage of PLM killing caused by the control aptamer was

probably due to the toxicity resulting from the expression of high concentrations of RNA in these neurons.

To determine if R9-2 and R9-7 killed PLM neurons through the *C. elegans* programmed cell death pathway, we crossed the high-copy number transgenes expressing R9-2 or R9-7 into the *ced-3(n717)* mutant animals, which are defective in almost all programmed cell death. As shown in Table 2, PLM deaths induced by either R9-2 or R9-7 were significantly inhibited in *ced-3(n717)* animals, suggesting that the CED-3 caspase activity is important for the killing activities of these two aptamers. The inhibition of the aptamer cell killing activity by the *ced-3(n717)* loss-of-function mutation was comparable to the inhibition of the EGL-1 killing activity by the *ced-3(n717)* mutation. Taken together, these results suggest that CED-9 aptamers induce ectopic PLM neuron deaths through the CED-3 caspase and the *C. elegans* apoptotic program.

Discussion

In an effort to isolate small molecular compounds that can modulate the activities of the Bcl-2 family proteins, we used the SELEX strategy to identify small RNA molecules that bind to the *C. elegans* Bcl-2 homologue CED-9 with high binding affinity and specificity. We have isolated and characterized five RNA aptamers for CED-9, which can be categorized into three groups based on their secondary structures. Detailed biochemical analyses of two of these aptamers, R9-2 and R9-7, indicate that they can form ternary complexes with CED-9 and EGL-1, suggesting that they bind to distinct surface areas on CED-9 from EGL-1. In contrast, another pro-apoptotic protein CED-4 could not form ternary complex with CED-9 and its aptamers (Fig. 3C). Analyses of the

interactions between R9-2 or R9-7 and several CED-9 mutants that are defective in binding to CED-4 revealed that these two aptamers and CED-4 share overlapping CED-9 binding sites (Fig. 4). Importantly, overexpression of R9-2 or R9-7 induced robust ectopic cell killing that is dependent on the CED-3 caspase activity, suggesting that they are true small molecule cell death inducers. The cell killing effect of R9-2 or R9-7 is likely due to their interference with CED-9/CED-4 interaction *in vivo*, which may prevent the sequestering of CED-4 to mitochondria by CED-9 and thus trigger apoptosis in those cells where the aptamers are expressed. The requirement of the CED-3 caspase activity for the cell killing activity of CED-9 aptamers is consistent with this possibility and suggests that these aptamers act upstream of *ced-3* to induce cell killing.

Since *in vitro* selected RNA aptamers usually bind to specific domains of their target proteins with high affinity and specificity, they can often be used to probe the function of a specific protein domain or to discriminate the functions of highly homologous proteins. For example, an *in vitro* selected RNA aptamer was used to discriminate the roles of two highly homologous proteins, cytohesin 1 and cytohesin 2, in regulating gene expression in response to serum stimulation (28). This aptamer binds to the N-terminal segment of cytohesin-2 specifically and can down-regulate expression of genes mediated through its serum response element and reduce mitogen-activated protein (MAP) kinase activation in HeLa cells, suggesting a specific role of cytohesin-2 but not cytohesin-1 in serum-mediated transcriptional activation in nonimmune cells. In our study, two aptamers that we isolated specifically recognize potential CED-4 binding sites on CED-9 without interfering with the binding of EGL-1 to CED-9. These aptamers can be very useful reagents for probing different functional domains of CED-9 in regulating

programmed cell death in *C. elegans*. Isolation of aptamers for other key cell death regulators in *C. elegans* will provide another powerful tool to facilitate the understanding of cell death activation in *C. elegans* and the characterization of poorly understood cell death regulators such as the pro-apoptotic protein CED-4.

Increasing evidence has shown that RNA aptamers are good lead compounds for developing diagnostic or therapeutic agents for treating human diseases (15). As a model experimental organism, *C. elegans* is being used to screen for compounds in drug development (39). The conservation of cell death pathways between nematodes and humans indicates that *C. elegans* can probably be used as an animal system to search for compounds that can modulate apoptosis in vivo. In both nematodes and humans, CED-9, Bcl-2, and Bcl-xL are the major cell death inhibitors and share similar protein structures (31, 40, 41). Furthermore, Bcl-2 can partially substitute for the function of CED-9 in *C. elegans*, suggesting that they may interact with the same cell death regulators and share crucial functional domains (12, 42). Thus our successful isolation and characterization of potent CED-9 aptamers not only can provide important insights into how Bcl-2 proteins may interact with other cell death factors to control the activation of apoptosis but also suggest that a similar strategy can be applied to isolate aptamers for Bcl-2, Bcl-xL, and other Bcl-2 family proteins. Elevated expression of Bcl-2, Bcl-xL and other Bcl-2 family proteins has been implicated in contributing to the development of a wide variety of human cancers and human diseases (10). Potent RNA aptamers specific for Bcl-2, Bcl-xL, or other Bcl-2 family proteins will be useful not only for studying how Bcl-2 family proteins regulate activation of apoptosis but also for developing new diagnostic reagents

or therapeutic drugs to detect or treat those human diseases caused by abnormal apoptosis.

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Table 1. Selected aptamer sequences for CED-9

Clone	(T7) AGGGAGGACGATGCG-N ₄₉ -CAGACGACGGA	Frequency
Group A		
R8-20	GGGTGGTCGCTTATCCGCATAGAGGTTTACGACTTCGGAGACTGCCGATA	3/30
R9-7	GGATGGACGCTTATCCGCATAGAGGTTTACTACTTCGGAGACTGCCGATA	9/30
R9-4	GGAGTCATGGCGCATAGGTAGCTTGTATGCTGCCAGAGACTGCCCTGTGA	8/30
Group B		
R9-2	GGGTGCTTCGAGCGTAGGAAGAAAGCCGGGGGCTGCAGATAATGTATAGC	2/30
Group C		
R9-8	GGTTGCCACGTTTATGTACAGAGACCGCCTCGGGAATATGACGCGCAGTA	5/30

The sequences shown are cDNA sequences for the corresponding RNA aptamers. T7 indicates the T7 promoter sequence (AATACGACTCACTATAG). Frequency indicates that the number of clones showing the same Aci I digestion pattern.

Table 2. Overexpression of CED-9 aptamers induces ectopic cell killing in PLM touch receptor neurons.

Transgene	PLM survival (%)				
	Low concentration (10 ng/μl)		High concentration (50 ng/μl)		
	Array	<i>bzIs8</i>	Array	<i>bzIs8</i>	<i>ced-3(n717); bzIs8</i>
None		100		100	100
P_{mec-7} R9-2	1	68	1	14	73
	2	74	2	23	89
P_{mec-7} R9-7	1	83	1	20	85
	2	85	2	19	75
Control aptamer	1	98	1	83	ND
	2	93	2	90	ND
P_{mec-7} <i>egl-1</i>			1	8	100
			2	7	97

CED-9 aptamer expression constructs were injected into a *C. elegans* strain (*bzIs8*) together with a co-injection marker pRF4 (50 μg/ml), which causes a Roller phenotype. $P_{mec-7egl-1}$ construct (25 μg/ml) was injected with $P_{ord-2rfp}$ (25 μg/ml), which directs RFP expression in a few head neurons. *bzIs8* is an integrated transgene containing a $P_{mec-4gfp}$ construct, which directs GFP expression in six *C. elegans* touch receptor neurons and allows scoring of the PLM neurons. Each numbered array represents an independent transgenic line. Thirty transgenic animals (animals

with a Roller phenotype or RFP expression in head neurons) from each line were scored for PLM survival using a fluorescent Nomarski microscope. ND, not determined.

Figure Legends

Fig. 1. Characterization of CED-9 aptamers. (A) Binding of aptamers to CED-9. ^{32}P -labeled aptamer (approximately 300cpm) was incubated with increasing amounts of CED-9(1-250)-His₆ as indicated at 30°C for 30 min and resolved on 7.5% native polyacrylamide gels. (B) Predicted secondary structures of CED-9 aptamers.

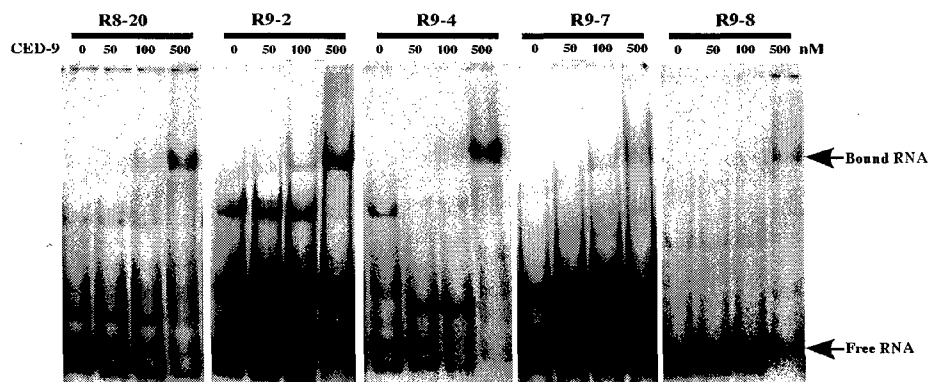
Fig. 2. Aptamers R9-2 and R9-7 compete with each other for binding to CED-9. (A) Approximately 8 nM of ^{32}P -labeled R9-2 was incubated with CED-9(1-250)-His₆ (500nM) in the presence of increasing concentrations (4, 8, 16, 32, 64, and 128 nM) of unlabeled R9-7 at 30°C for 30 min and separated with 7.5% native polyacrylamide gel. (B) Approximately 8 nM of ^{32}P -labeled R9-7 competes with increasing concentrations (4, 8, 16, 32, 64, and 128 nM) of unlabeled R9-2 for binding to CED-9.

Fig. 3. EGL-1 but not CED-4 forms a ternary complex with CED-9 and its aptamers. (A) ^{32}P -labeled R9-2 was incubated with CED-9(1-250)-His₆ (500 nM) in the presence of increasing concentrations (0, 0.5, 1.0, and 1.5 μM , lane 3-6) of GST-EGL-1. The reactions were resolved by EMSA. (B) R9-7 forms a ternary complex with CED-9 and EGL-1 as assayed in (A). (C) R9-2 binding to CED-9 in the presence of increasing concentrations of CED-4 as assayed in (A).

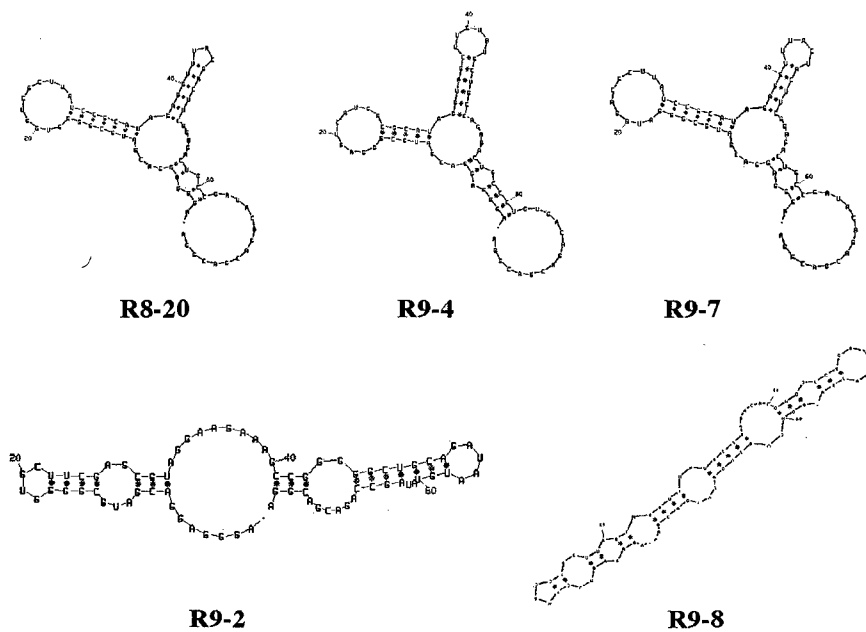
Fig. 4. CED-9 aptamers and CED-4 share overlapping CED-9 binding sites. (A) Binding of R9-2 and R9-7 aptamers to CED-9 mutants. 2.5 μM of wild-type or mutant GST-CED-9 proteins were incubated with ^{32}P -labeled aptamers and assayed with EMSA as

describe in Figure 1. “+++” indicates a strong aptamer/CED-9 binding; “+” indicates a significantly reduced binding between an aptamer and a CED-9 protein; “-” indicates no obvious binding between an aptamer and a CED-9 protein. “*” indicates the interactions between CED-9 mutants and CED-4 as described by Yan et al (31). (B) Interactions between CED-9 mutants and EGL-1. Equal amounts of wild-type or mutant GST-CED-9 proteins were incubated with ³⁵S-Methionine-labeled EGL-1 and pulled down using glutathione-Sepharose beads as described (37) and viewed with phosphorimager.

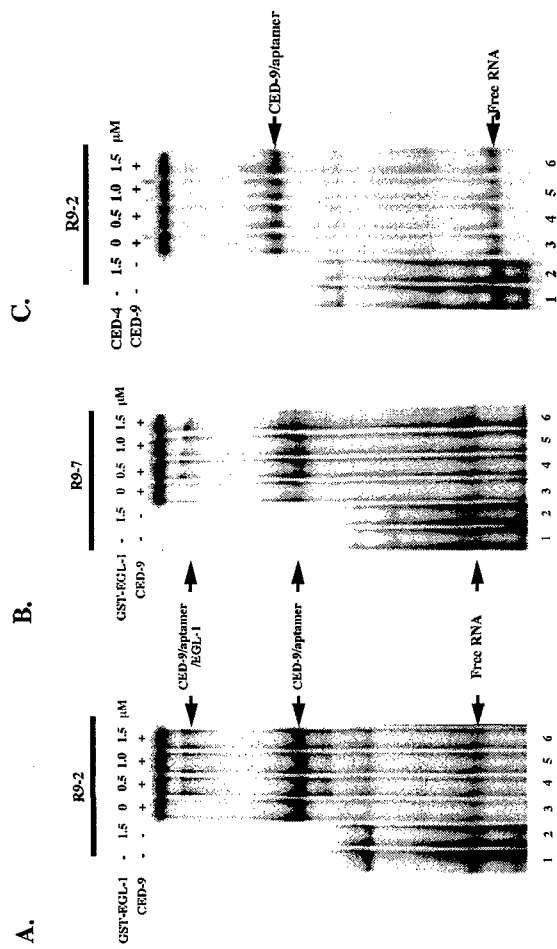
A.



B.







A.

	R9-2	R9-7	CED-4*
WT	+++	+++	+++
EPR75-77RGE	-	-	+++
D79K, K81R	+++	+++	+++
P103G, G104E	+++	+	+++
S107G, G108K	+++	+++	+++
EQ136-137KA	-	+	+++
KKH125-128EEA	-	+	+++
NAQ158-160AGA	-	-	+
Y201D	+	-	+
RN211-212EG	-	-	+

B.

